mesothelin was observed in 16 cases, while the cytoplasmic expression was detected in 42 tumours, which included the 9 cases of 'positive for both luminal membrane and cytoplasm' (Figure 2). The detailed clinicopathological information of 16 cases with mesothelin luminal membrane expression was summarised in Supplementary Table 2. We never detected the mesothelin expression in the non-cancerous lesions (data not shown). The statistical analysis revealed that the incidence of mesothelin expression was only correlated with lymph-node metastasis ($P=0.028$), while the incidence of luminal membrane expression of mesothelin was correlated with pT factor ($P=0.0019$), lymph-node metastasis ($P=0.0029$), clinical stage ($P=0.0002$), lymphatic permeation ($P=0.0019$), blood vessel invasion ($P=0.0098$), and recurrence ($P<0.0001$). There were no significant correlations between mesothelin cytoplasmic expression and clinicopathological parameters (Table 1).

Survival analysis associated with mesothelin expression

The analysis for patients' overall survival denoted that the group of 'luminal membrane positive' for mesothelin indicated a significantly unfavourable outcome compared with the group of 'luminal membrane negative' ($P<0.001$). On the other hand, the pure mesothelin expression regardless of the localisation, and also 'cytoplasmic expression' were not correlated with the overall survival of the patients (Figure 3). To confirm the mesothelin expression as an independent prognostic factor, we performed the univariate analysis of the 110 gastric cancers using the Cox proportional hazards model, and obtained the result that pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression were significantly correlated with the risk of cancer death (Table 2). Furthermore, to exclude the possible interference of any other factors, the multivariate analysis was performed including pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression. Interestingly, the luminal membrane expression of mesothelin was an independent predictor of overall survival for gastric cancer patients as well as clinical stage and lymphatic permeation (Table 3).

Mesothelin expression in metastatic lymph nodes

As shown above, luminal membrane expression of mesothelin was correlated with lymphatic permeation and lymph-node metastasis; thus, we analysed the expression pattern of mesothelin in 35 out of 37 cases of lymph-node metastasis by immunohistochemistry, in which the tissue blocks of metastatic lymph node were available (Supplementary Figure 2). Interestingly, the incidence of luminal membrane positive including expression in both membrane and cytoplasm was increased in metastatic lymph nodes (51.4%; 18 out of 35) compared with primary lesions (31.4%; 11 out of 35). Moreover, in 4 cases out of 14 mesothelin-negative cases in primary lesion, luminal membrane expression of mesothelin was observed. These results support our idea that luminal membrane expression of mesothelin is associated with the malignant behaviour of tumour cells.

DISCUSSION

In this study, we demonstrated that the luminal membrane expression of mesothelin in gastric cancer was associated with unfavourable clinical outcome in patients after surgery. The univariate analysis indicated that the luminal membrane expression of mesothelin was also correlated with lymph-node metastasis, clinical stage, lymphatic permeation, blood vessel invasion, residual tumour, and recurrence, although a luminal membrane expression of mesothelin remained a statistically independent factor for favourable patient outcome after the multivariate analysis. Our result that total mesothelin expression including the case of exclusive cytoplasmic expression did not correlate with patients' prognosis will explain the discrepant previous report in which mesothelin expression correlates with prolonged patient survival in gastric cancer (Baba *et al*, 2011). We therefore emphasise that membrane-localised mesothelin might have an important role in the development of gastric cancer.

The full length of human *mesothelin* gene codes the primary product being a 71-kDa precursor protein. It can be

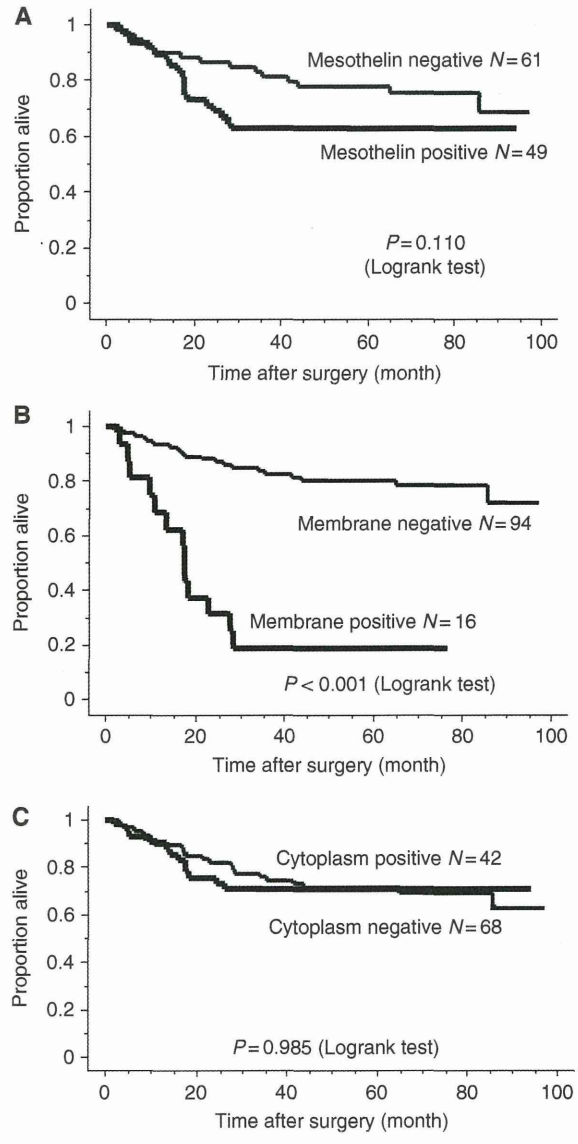

Figure 3 Overall survival for patients with gastric cancer after surgical therapy stratified by the status of mesothelin expression (**A**), mesothelin luminal membrane expression (**B**), and mesothelin cytoplasmic expression (**C**), respectively. The group of 'luminal membrane positive' represented a statistically significantly unfavourable outcome compared with the group of 'luminal membrane negative' (**B**: $P<0.001$). On the other hand, both total expression (**A**) and cytoplasmic expression of mesothelin (**C**) were not correlated with overall survival of the patients.

Table 2 Univariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma

Factor	N	P	RR (95% CI)
1. *Histological classification*			
por2-sig	62	0.89	1
Others	48		0.954 (0.478–1.903)
2. *pT factor*			
pT1	62	<0.0001	1
pT2-4	48		13.354 (4.679–38.113)
3. *pN factor*			
Positive	73	<0.0001	1
Negative	37		9.301 (4.147–20.860)
4. *pStage*			
I, II	80	<0.0001	1
III, IV	30		18.837 (8.032–44.179)
5. *Lymphatic permeation*			
Positive	62	<0.0001	1
Negative	48		18.529 (5.637–60.534)
6. *Blood vessel permeation*			
Positive	69	<0.0001	1
Negative	41		11.493 (4.722–27.971)
7. *Mesothelin expression*			
No	61	<0.0001	1
Yes	49		1.749 (0.874–3.500)
8. *Luminal membrane expression*			
No	94	<0.0001	1
Yes	16		7.205 (3.489–14.877)
9. *Cytoplasmic expression*			
No	68	0.98	1
Yes	42		1.007 (0.493–2.055)

Abbreviation: CI = confidence interval. RR indicates relative risk/hazard ratio.

Table 3 Multivariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma

Factor	P	RR (95% CI)
1. *pT factor*		
pT1 *vs* pT2–4	0.35	2.497 (0.374–16.660)
2. *pN factor*		
Positive *vs* Negative	0.060	3.532 (0.946–13.181)
3. *pStage*		
I, II *vs* III, IV	0.0003	12.336 (2.533–60.069)
4. *Lymphatic permeation*		
Positive *vs* Negative	0.0043	11.996 (2.180–65.996)
5. *Blood vessel permeation*		
Positive *vs* Negative	0.29	2.091 (0.533–8.195)
6. *Luminal membrane expression*		
No *vs* Yes	0.0073	2.969 (1.341–6.573)

Abbreviation: CI = confidence interval. RR indicates relative risk/hazard ratio.

physiologically cleaved by some furin-like proteases into a 40-kDa C-terminal fragment that remains membrane bound, and a 31-kDa N-terminal fragment, which is secreted into the blood (Chang and Pastan, 1996). The C-terminal 40-kDa fragment is referred to as mesothelin, which is attached to the cell membrane by a GPI anchor (Chang and Pastan, 1996; Hassan *et al*, 2004). The 5B2 anti-mesothelin antibody (Novocastra Laboratory Vision BioSystems, Boston, MA, USA), which we employed here for IHC, can detect the 71-kDa precursor protein and also the 40-kDa C-terminal fragment (Inami *et al*, 2008); therefore, we could not decide which form of mesothelin has a pivotal role in malignant behaviour of gastric cancer cells. Recent studies reported that mesothelin is not only associated with increased cell proliferation and with the migration of pancreatic cancer cells *in vitro* (Bharadwaj *et al*, 2008; Li *et al*, 2008), but also contributes to tumour progression *in vivo* (Li *et al*, 2008). Mesothelin inhibits paclitaxel-induced apoptosis through concomitant activation of phosphoinositide-3-kinase (PI3K) signalling in the regulation of Bcl-2 family expression (Chang *et al*, 2009), and induces the activation of signal transducer and activator of transcription (Stat) 3, which leads to increased expression of cyclin E and makes pancreatic cancer cells proliferate faster (Bharadwaj *et al*, 2008). In addition, mesothelin-activated nuclear factor-kappaB (NF-κB) induces elevated interleukin (IL)-6 expression, which acts as a growth factor to support pancreatic cancer cell survival/proliferation through a novel auto/paracrine IL-6/soluble IL-6R trans-signalling (Bharadwaj *et al*, 2011a, b). Our study provided a new aspect that luminal membrane expression of mesothelin is associated with the malignant behaviour of tumour cells, such as depth of tumour invasion and vascular invasion, although it remains necessary to clarify the biological function of the 71-kDa mesothelin precursor and/or 40-kDa mesothelin protein in *in-vitro* and *in-vivo* studies, including the processing system by furin-like proteases.

In terms of discovering the clinicopathological parameters for gastric cancer, there are many previous studies demonstrating the prognostic significance of various molecules, such as epidermal growth factor receptor and c-erB-2 (HER-2). These molecules also could be of unique significance as the indicators of eligibility to specific molecular targeting therapies, because most of them are located in the cell membrane as the useful targets for the molecular targeted drugs such as antibody drugs. We believe that the immunohistochemical evaluation for luminal membrane expression of mesothelin in gastric cancer would be of clinical benefit not only as a prognostic factor but also as a predictive factor for the eligibility to mesothelin-targeting therapies in the future (Hassan *et al*, 2004, 2007a, b, c, 2010; Hassan and Ho, 2008; Li *et al*, 2008; Inami *et al*, 2009).

In conclusion, we demonstrated the clinicopathological significance of the luminal membrane expression of mesothelin in gastric cancer as an independent prognostic factor, although additional studies to increase the number of the cases for luminal membrane expression ($n=16$) might be required for further confirmation. The immunohistochemical examination of mesothelin expression in surgically resected tumour specimens should be clinically useful for prognostication and for decision making about further treatment procedures after surgical therapy in patients with gastric cancer.

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ORIGINAL ARTICLE

A rare point mutation in the Ras oncogene in hepatocellular carcinoma

Akinobu Taketomi · Ken Shirabe · Jun Muto · Shohei Yoshiya · Takashi Motomura · Yohei Mano · Tohru Ikegami · Tomoharu Yoshizumi · Kenji Sugio · Yoshihiko Maehara

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Abstract
Purpose The Ras gene is one of the oncogenes most frequently detected in human cancers, and codes for three proteins (K-, N-, and H-Ras). The aim of this study was to examine the mutations in codons 12, 13 and 61 of the three Ras genes in cases of human hepatocellular carcinoma (HCC).
Methods Paired samples of HCC and corresponding non-malignant liver tissue were collected from 61 patients who underwent hepatectomy. A dot-blot analysis was used to analyze the products of the polymerase chain reaction (PCR) amplification of codons 12, 13, and 61 of K-, N- and H-Ras for mutations.
Results Only one mutation (K-Ras codon 13; Gly to Asp) was detected among the 61 patients. Interestingly, this patient had a medical history of surgery for both gastric cancer and right lung cancer. No mutations were found in codons 12 and 61 of K-Ras or codons 12, 13 and 61 of the N-Ras and H-Ras genes in any of the HCCs or corresponding non-malignant tissues.
Conclusions These findings indicated that the activation of Ras proto-oncogenes by mutations in codons 12, 13, and 61 does not play a major role in hepatocellular carcinogenesis.

Keywords Ras · Mutation · Hepatocellular carcinoma · Sorafenib

A. Taketomi (✉) · K. Shirabe · J. Muto · S. Yoshiya · T. Motomura · Y. Mano · T. Ikegami · T. Yoshizumi · K. Sugio · Y. Maehara
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Japan
e-mail: taketomi@med.hokudai.ac.jp; taketomi@surg2.med.kyushu-u.ac.jp

Abbreviations

Asp	Asparagine
Glu	Glutamate
Gly	Glycine
HCC	Hepatocellular carcinoma
Lys	Lysine
PCR	Polymerase chain reaction
TTP	Time to progression
Val	Valine

Introduction

Hepatocellular carcinoma (HCC) is a global health problem, accounting for more than 80 % of all primary liver cancers, and is one of the most common malignancies worldwide [1]. Most patients with HCC also present with concomitant cirrhosis, which is the major clinical risk factor for hepatic cancer, and results from alcoholism or infection with the hepatitis B or hepatitis C virus. Primary liver malignancies (95 % of which are HCC) are the third and fifth leading causes of cancer death among males and females, respectively, in Japan [2]. Both liver resection and liver transplantation are potentially curative treatments for HCC [3–5]. Although other treatment options, including percutaneous radiofrequency ablation or chemolipiodolization are also available, there is no standard systemic therapy for advanced cases.

Sorafenib (BAY 43-9006, Nexavar) is a novel oral kinase inhibitor that targets multiple tyrosine kinases in vivo and in vitro, and is widely used for HCC [6]. The main targets of sorafenib are the receptor tyrosine kinase pathways which are frequently deregulated in cancer, such as the Ras pathway. The Ras pathway represents a dominant signaling network promoting cell proliferation and

survival. The binding of different growth factors (e.g. epidermal growth factor: EGF) to their receptors (e.g. epidermal growth factor receptor: EGFR) induces the activation of Ras, which in turn activates c-raf, MEK and ERK. Phosphorylated ERK in the nucleus activates transcription factors that regulate the expression of genes involved in cell proliferation and survival.

A phase II trial involving 137 patients with advanced HCC showed that sorafenib induced partial responses in less than 5 % of patients, but the observed median survival of 9.2 months with a median time to progression of 5.5 months was classified as evidence of potential clinical benefit, since the expected median survival of these patients is 6 months [7]. Consequently, a large phase III clinical trial (SHARP) was conducted in 602 patients with advanced HCC. The results showed a 31 % decrease in the risk of death, with a median survival of 10.6 months in the sorafenib arm versus 7.9 months for placebo [8]. In addition, sorafenib showed a significant benefit in terms of the time to progression (TTP) as assessed by independent radiological review, with a median TTP of 5.5 months for the sorafenib and 2.8 months for the placebo arm.

Because Ras is one of the targets of sorafenib, it is important to determine whether mutations in the Ras gene result in the activation of the Ras/MAPK pathway in human HCCs. However, the relationship between Ras mutations and human HCC has not been fully evaluated. The present study was designed to investigate K-, N- and H-Ras (*KRAS, NRAS, HRAS*) somatic mutations in human HCC.

Materials and methods

Patients and tumor samples

Tumor tissue samples were obtained from 61 Japanese patients who underwent surgical resection for HCC during the period between December 1989 and April 1992 in the Department of Surgery and Science, Kyushu University Hospital, Fukuoka, Japan. Surgically resected tissue samples were frozen at −80 °C immediately after resection and were stored until use in this study. Written informed consent was obtained from all patients examined, and the current study was approved by the Kyushu University ethics committee.

DNA preparation and detection of Ras point mutations

High molecular weight DNA was isolated from frozen tumor samples, as described elsewhere [9]. Selective amplification of the Ras gene sequence was done using a PCR technique. The nucleotide sequences of the primers used are listed in Table 1. The PCR was performed at

Table 1 Ras gene primers used in this study

Gene/codon	Length (bp)	Sequence	
KRAS/12, 13	108	Forward	GACTGAATATAAACTTGTGG
		Reverse	CTATTGTTGGATCATATTCG
KRAS/61	128	Forward	TTCCTACAGGAAGCAAGTAG
		Reverse	CACAAAGAAAGCCCTCCCCA
HRAS/12, 13	63	Forward	GACGGAATATAAGCTGGTGG
		Reverse	TGGATGGTCAGCGCACTCTT
HRAS/61	73	Forward	AGACGTGCCTGTTGGACATC
		Reverse	CGCATGTACTGGTCCCGCAT
NRAS/12, 13	109	Forward	GACTGAGTACAAACTGGTGG
		Reverse	CTCTATGGTGGGATCATATT
NRAS/61	103	Forward	GGTGAAACCTGTTTGTTGGA
		Reverse	ATACACAGAGGAAGCCTTCG

bp base pairs

96 °C to denature the DNA (1 min), at 55 °C (*NRAS*), 57 °C (*KRAS*), 62 °C (*HRAS*) to anneal the primer (30 s), and at 72 °C to synthesize DNA (10 s to 1 min) using Taq DNA polymerase for 35–40 cycles in a DNA thermal cycler (Perkin-Elmer-Cetus). Amplified DNA samples were spotted onto nylon membranes (Hybond N+) for the hybridization analysis. All of the DNA isolated from the 61 tumor samples and the corresponding non-malignant liver tissues were screened for activated point mutations in codons 12, 13, and 61 of all three Ras genes using an oligonucleotide specific for the different sequences. The filters were prehybridized for 1 h at 55 °C in solution A (3.0 M tetramethylammonium chloride, 50 mM Tris-HCI, 2 HIMEDTA, 0.1 % SDS, 5× Denhardt's solution, 100 fg/ml denatured herring sperm DNA), and hybridized for 1 h at 55 °C in the same solution with 5 pmol ^{32}P-labeled probe. These filters were washed twice in 0.3 M NaCl, 0.02 M NaH2PO4, 2 mM EDTA and 0.1 % SDS at room temperature for 5 min, and in solution A without Denhardt's solution and herring sperm DNA, once for 5 min at room temperature and twice for 10 min at 60 °C. These filters were then exposed to Kodak XAR5 film. Human cancer cell lines carrying Ras genes mutations were used as positive controls. The colon cancer cell lines: SW620 (*KRAS* codon 12 GTT:Val), LSI80 (*KRAS* codon 12 GAT:Asp), and LOVO (*KRAS* codon 13 GAC:Asp) were obtained from the Japanese Cancer Research Resources Bank, and KMS4 (*KRAS*s codon 12 TGT:Cys) was provided by Dr. Sugio (Institution?).

Results

The age of the 61 patients ranged from 43 to 79 years (average, 64.1 years), and 46 were males and 15 were

females. The positive rate of hepatitis surface B antigen was 12.9 %, and the positive rate of anti-hepatitis C virus antibody was 72.7 %. The mean tumor size was 4.47 cm.

One of the 61 HCCs (1.6 %) carried a point mutation, which was a G to A transition at codon 13 of the *KRAS* gene (Fig. 1). DNA extracted from the corresponding non-malignant liver tissue had the normal codon, suggesting that mutational activation of K-ras was involved in the malignant transformation in this case. This patient was positive for anti-hepatitis C virus antibodies, and was classified to have Child-Pugh A disease. The diameter of this patient's tumor was 12 cm, and the tumor was composed of well to moderately differentiated hepatocellular carcinoma. Interestingly, this patient had undergone surgery for gastric cancer 18 years before and lung cancer 12 years before the surgery for HCC.

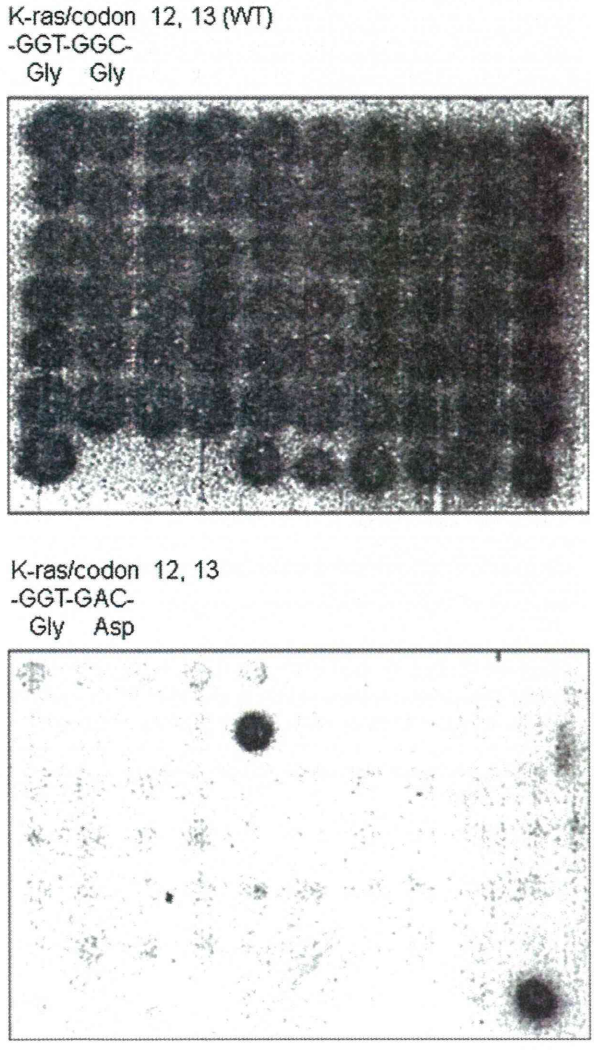

Fig. 1 Detection of a *KRAS* gene mutation in a patient with hepatocellular carcinoma. PCR-amplified DNA from 61 tumor samples was dotted onto nylon membranes and hybridized to a ^{32}P-labeled oligonucleotide probe. *WT* wild type *KRAS*

No mutational activation was found in codons 12 and 61 of *KRAS* or codons 12, 13 and 61 of the *NRAS* and *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples.

Discussion

This study examined 61 HCC tissues and their corresponding non-malignant liver tissues for a somatic mutation in codons 12, 13, and 61 of the *KRAS*, *HRAS*, or *NRAS* genes, which are known hot spots in various malignancies. However, the study showed the only one of the 61 HCCs (1.6 %) had a somatic mutation in codon 13 of the *KRAS* gene, indicating that Ras gene mutations do not appear to be related to the pathogenesis of most HCCs.

There have been several reports with small sample sizes regarding Ras gene mutations in HCC (Table 2). Most have reported that somatic mutations of the Ras gene in HCCs are uncommon, similar to the current study. Tsuda et al. [10] found only two tumors with Ras point mutations in surgically resected specimens from 30 HCC patients. In their patients, codon 12 of *KRAS* was altered from GGT, coding for Gly, to GTT, coding for Val in one case, and codon 61 of *NRAS* was altered from CAA, coding for Glu, to AAA, coding for Lys, in the other case. Tada et al. analyzed the mutations of the three Ras genes in 23 primary hepatic malignant tumors (12 hepatocellular carcinomas, nine cholangiocarcinomas, and two hepatoblastomas). Point mutations in *KRAS* codon 12 or *KRAS* codon 61 were found in 6 of the 9 cholangiocarcinomas. In contrast, there were no point mutations in any of 12 HCCs or two hepatoblastomas in codons 12, 13, or 61 of the Ras genes. The authors concluded that Ras gene mutations are not related to the pathogenesis of HCC, but play an important role in pathogenesis of cholangiocarcinoma.

Sorafenib is the first molecule with specific targets involved in the pathogenesis of HCC that has become available for routine clinical use. It is an orally applicable

Table 2 Reported Ras gene mutations in HCC patients

Author [references]	No. of patients	Ras gene mutation		
		KRAS	*NRAS*	*HRAS*
Tsuda et al. [10]	30	1 (codon 12)	1 (codon 61)	0
Tada et al. [14]	12	0	0	0
Ogata et al. [15]	19			2
Challen et al. [16]	19	1 (codon 61)	3 (codon 61)	0
Leon et al. [17]	12	1 (codon 61)	0	0
This study	61	1 (codon 13)	0	0

multi-kinase inhibitor that acts by blocking tumor cell proliferation and angiogenesis through the inhibition of serine/threonine kinases [11]. Sorafenib can increase survival by up to 3 months in patients with advanced HCC and acceptable liver function [8]. On the other hand, severe side effects have been reported with sorafenib, including hand-foot skin reactions or liver dysfunction [7, 8]. Therefore, it is important to identify prognostic markers and to establish the proper selection criteria for using sorafenib. Mutations of the Ras genes in cases of HCCs were systemically evaluated in this study because the Ras signaling pathway is the main target of sorafenib. The results indicated that mutational activation of Ras genes is uncommon in the pathogenesis of HCCs. Caraglia et al. [12] reported that the presence of phosphorylated ERK activity in peripheral blood mononuclear cells is valuable for predicting the response to sorafenib therapy in HCC patients. An in vitro study confirmed that phosphorylated ERK was a potential biomarker predicting the sensitivity of HCC to sorafenib [13]. Therefore, a mutation in the RAF/MEK/ERK pathway may be involved in the drug resistance to sorafenib, rather than a Ras mutation.

In summary, only one of 61 HCCs (1.6 %) in the present study carried a point mutation, which was a G to A transition in codon 13 of the *KRAS* gene. No mutational activation was found in codons 12 and 61 of *KRAS* or in codons 12, 13 and 61 of the *NRAS* or *HRAS* genes in any of the HCCs or corresponding non-malignant tissue samples. These findings suggested that Ras gene mutations are not related to the pathogenesis of most HCCs. The signaling pathways downstream of Ras should be examined to identify markers to predict a response to sorafenib.

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Conflict of interest None of the authors has any conflict of interest.

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Tumor-Associated Macrophage Promotes Tumor Progression via STAT3 Signaling in Hepatocellular Carcinoma

Yohei Mano[a, b] Shinichi Aishima[a] Nobuhiro Fujita[c] Yuki Tanaka[a]
Yuichiro Kubo[a] Takashi Motomura[b] Akinobu Taketomi[b] Ken Shirabe[b]
Yoshihiko Maehara[b] Yoshinao Oda[a]

Departments of [a]Anatomic Pathology, [b]Surgery and Science, and [c]Clinical Radiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

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Hepatocellular carcinoma · STAT3 · Macrophage

Abstract
Objective: Signal transducer and activator of transcription 3 (STAT3) is activated in hepatocellular carcinoma (HCC), and tumor-associated macrophage plays an important role in tumor progression. Therefore, we examined STAT3 activation, cytokine expression and infiltration of tumor-associated macrophages in resected HCCs as well as the alteration of cell growth and migration by cytokine stimulation in HCC cell lines. ***Methods:*** Immunohistochemical staining of phosphorylated STAT3 (pSTAT3), CD163, interleukin (IL)-6, Ki-67 and Bcl-XL was performed for 101 cases of resected HCC, and correlations between pSTAT3 staining and clinicopathological findings were analyzed. In HCC cell lines (PLC/PRF/5 and Huh7), cell proliferation and migration by IL-6 stimulation and S3I-201 (STAT3 inhibitor) treatment were analyzed. ***Results:*** In HCC specimens, the pSTAT3-positive group showed high levels of α-fetoprotein ($p = 0.0276$), large tumor size ($p = 0.0092$), frequent intrahepatic metastasis ($p = 0.0214$), high Ki-67 ($p = 0.0002$) and Bcl-XL ($p = 0.0001$), poor prognosis ($p = 0.0234$), and high recurrence rate ($p = 0.0003$). CD163-positive cells were frequently observed in the pSTAT3-positive group ($p = 0.0013$). In two HCC cell lines, IL-6 stimulation promoted cell proliferation and migration via the STAT3 phosphorylation, and S3I-201 inhibited this activation. ***Conclusions:*** STAT3 activation was correlated with aggressive behavior of HCC and may be mediated via tumor-associated macrophage. We expect that STAT3 signaling and tumor-associated macrophages can be attractive therapeutic targets in HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer in the world [1]. Although surgical therapies for HCC have progressed and outcomes of HCC have improved, HCC still often recurs after surgery [2, 3]. Sorafenib, one of the molecular targeted therapies, was reported to show activity against unresectable HCCs;

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Yoshinao Oda, PhD
Department of Anatomic Pathology
Graduate School of Medical Sciences, Kyushu University
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 (Japan)
E-Mail oda@surgpath.med.kyushu-u.ac.jp